

Description

METHOD FOR PROGNOSTIC EVALUATION OF CARCINOMA USING ANTI-P-LAP ANTIBODY

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Technical Field

The present invention relates to a reagent for diagnosis and/or prognostic evaluation of carcinoma, comprising
10 determination of placental leucine aminopeptidase (P-LAP) which is a diagnostic and/or prognostic factor in carcinoma, a method for determination of P-LAP in carcinoma tissues, and a method for prognostic evaluation of carcinoma.

15 Background Art

The number of carcinoma patient has been increasing these days. For example, endometrial endometrioid adenocarcinoma, including endometrial carcinoma and endometrioid
adenocarcinoma, is a common gynecological malignancy in the
20 United States. Recently, the incidence of this disease is increasing in Japan. Also, it is known that a significant number of patients with early-stage endometrial endometrioid adenocarcinoma develop both localized recurrence and distant metastasis (Non-patent literature 1).

25 Meanwhile, for carcinoma patients who have undergone surgical operation, accurate prognostic evaluation in early stage is extremely important for proper post-operative treatment. For example, a chemotherapy having a risk of side effect should be avoided for patients with good prognosis who

do not need chemotherapy. On the other hand, appropriate treatment including chemotherapy should be provided to patients who are at high risk of recurrence of carcinoma or reoperation in order to prevent recurrence of carcinoma or to avoid
5 reoperation. Hence, various researches have been conducted to find prognostic factors, however, a credible prognostic factor has not yet being discovered.

With regard to endometrial endometrioid adenocarcinoma, for example, several clinicopathological indicators are
10 currently used for prognosis, such as the surgical stage of disease, lymph node involvement, myometrial infiltration, histological cell types, differentiation grade of carcinoma, intraperitoneal spread, cervical extension, vascular invasion and the like (non-patent literature 2). However, it cannot be
15 said that the clinicopathological indicators described in the literature are completely reliable indicators for prognosis of endometrial endometrioid adenocarcinoma.

On the other hand, P-LAP is a cell surface aminopeptidase, and is a synonym for oxytocinase (non-patent literature 3).
20 P-LAP is also referred to as insulin-regulated membrane aminopeptidase associated with the glucose transporter 4 (GLUT4) containing vesicles (non-patent literature 4). Recently, it was shown that P-LAP is present in both human endometrial endometrioid adenocarcinoma tissues and cells, and
25 that it works as a regulator of carcinoma cell growth (non-patent literature 5). Also, there has been reported the intriguing changes of P-LAP in normal endometrium during menstrual cycle (non-patent literature 6). However, it has not been known that P-LAP can be a prognostic factor in carcinoma.

(Non-patent literature 1) Am. J. Obstet. Gynecol., 1983;
146: 141-144

(Non-patent literature 2) Gynecol. Oncol., 1991; 40:
55-65

5 (Non-patent literature 3) Arch. Biochem. Biophys., 1992;
292: 388-392

(Non-patent literature 4) J. Biol. Chem., 1995; 270:
23612-23618

(Non-patent literature 5) Clin. Cancer Res., 2003; 9(4):
10 1528-1534

(Non-patent literature 6) J. Clin. Endocrinol. Metab.,
2002; 87: 1384-1389

Disclosure of the Invention

15 An object of the present invention is to provide a reagent
for diagnosis and/or prognostic evaluation of carcinoma.
Another object of the present invention is to provide a method
for determination of prognostic factors in carcinoma. A still
another object of the present invention is to provide a method
20 for prognostic evaluation of carcinoma.

The present inventors conducted various studies to solve
the aforementioned problems. As a result, they found that P-LAP
could be a prognostic factor in carcinoma, including
gynecological carcinoma such as endometrial endometrioid
25 adenocarcinoma, cervical carcinoma, ovarian carcinoma, etc.,
and thus completed the present invention.

Namely, the present invention relates to

(1) A reagent for diagnosis and/or prognostic evaluation
of carcinoma, which comprises an anti-P-LAP antibody as an

active ingredient;

(2) The reagent according to the above (1), wherein the carcinoma is gynecological carcinoma;

(3) The reagent according to the above (2), wherein the
5 gynecological carcinoma is endometrial endometrioid adenocarcinoma, cervical carcinoma or ovarian carcinoma;

(4) The reagent according to any one of the above (1) to (3), wherein the anti-P-LAP antibody is an anti-human P-LAP antibody;

10 (5) The reagent according to any one of the above (1) to (3), wherein the anti-P-LAP antibody is an anti-human P-LAP polyclonal antibody;

(6) A method for determination of P-LAP which is a prognostic factor in carcinoma, which comprises

15 (a) a step of contacting carcinoma tissues obtained from carcinoma patients with an anti-P-LAP antibody, and

(b) a step of measuring the intensity of the specific antigen-antibody reaction between P-LAP present in the carcinoma tissues and anti-P-LAP antibody;

20 (7) The method according to the above (6), wherein the carcinoma is gynecological carcinoma;

(8) The method according to the above (7), wherein the gynecological carcinoma is endometrial endometrioid adenocarcinoma, cervical carcinoma or ovarian carcinoma;

25 (9) The method according to any one of the above (6) to (8), wherein the anti-P-LAP antibody is an anti-human P-LAP antibody;

(10) The method according to any one of the above (6) to (8), wherein the anti-P-LAP antibody is an anti-human P-LAP

polyclonal antibody;

(11) A method for prognostic evaluation of carcinoma, which comprises

(a) a step of contacting carcinoma tissues obtained from
5 carcinoma patients with an anti-P-LAP antibody,

(b) a step of measuring the intensity of the specific antigen-antibody reaction between P-LAP present in the carcinoma tissues and anti-P-LAP antibody, and

(c) a step of correlating the intensity of the specific
10 antigen-antibody reaction with prognosis of carcinoma;

(12) The method according to the above (11), wherein the carcinoma is gynecological carcinoma;

(13) The method according to the above (12), wherein the gynecological carcinoma is endometrial endometrioid
15 adenocarcinoma, cervical carcinoma or ovarian carcinoma;

(14) The method according to any one of the above (11) to (13), wherein the anti-P-LAP antibody is an anti-human P-LAP antibody;

(15) The method according to any one of the above (11) to (13), wherein the anti-P-LAP antibody is an anti-human P-LAP
20 polyclonal antibody; and

(16) An immunoassay kit for determination of the amount of P-LAP present in carcinoma tissues obtained from carcinoma patients, which comprises an anti-P-LAP antibody and a marker
25 enzyme for determination of the amount of the P-LAP bound to the anti-P-LAP antibody.

An accurate prognostic evaluation of carcinoma can be achieved when the reagent of the present invention is used and the amount of P-LAP present in carcinoma tissues obtained from

patients with carcinoma, including gynecological carcinoma such as endometrial endometrioid adenocarcinoma, is determined. This makes it possible to provide postoperative patients with appropriate treatment.

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Brief Description of the Drawing

Fig. 1 shows preferential overexpression of P-LAP in human endometrioid adenocarcinoma tissues.

Fig. 2 shows correlation between P-LAP immunoreactivity
10 and disease-free interval in endometrial endometrioid adenocarcinoma patients.

Fig. 3 shows correlation between P-LAP immunoreactivity and disease-free interval in early stage endometrial endometrioid adenocarcinoma patients.

Fig. 4 shows correlation between P-LAP immunoreactivity
15 and disease-free interval in advanced stage endometrial endometrioid adenocarcinoma patients.

In the Fig. 1,

symbol A shows negative P-LAP immunoreactivity in grade
20 1 endometrioid adenocarcinoma tissues;

symbol B shows negative P-LAP immunoreactivity in grade 2 endometrioid adenocarcinoma tissues;

symbol C shows weakly positive P-LAP immunoreactivity in grade 1 endometrioid adenocarcinoma tissues;

symbol D shows strongly positive P-LAP immunoreactivity
25 in grade 1 endometrioid adenocarcinoma tissues;

symbol E shows strongly positive P-LAP immunoreactivity in grade 2 endometrioid adenocarcinoma tissues; and

symbol F shows strongly positive P-LAP immunoreactivity

in grade 3 endometrioid adenocarcinoma tissues.

Best Mode for Carrying Out the Invention

Anti-P-LAP antibody which is an active ingredient of the
5 reagent of the present invention is preferably an anti-human
P-LAP antibody. Also, the anti-human P-LAP antibody may be a
polyclonal antibody or a monoclonal antibody. Those
antibodies can be produced using P-LAP as antigen according to
conventional methods. For example, it is possible to produce
10 an anti-human P-LAP polyclonal antibody by the method described
in PLACENTA, Nakanishi et al., 2000, vol. 21, p.628-634.

Carcinoma tissues obtained from carcinoma patients are
used for prognostic evaluation of carcinoma using the reagent
of the present invention. Although there is no limitation on
15 the types of carcinoma, examples of carcinoma preferably
include gynecological carcinoma such as endometrial
endometrioid adenocarcinoma (including endometrial carcinoma
and endometrioid adenocarcinoma), ovarian carcinoma, cervical
carcinoma, etc.; pancreatic carcinoma; prostate carcinoma and
20 the like, more preferably gynecological carcinoma such as
endometrial endometrioid adenocarcinoma, cervical carcinoma,
ovarian carcinoma, etc.

The carcinoma tissues obtained from carcinoma patients
are fixed in formalin, embedded in paraffin and made into
25 sections of appropriate size to prepare carcinoma tissue
samples, and then the amount of P-LAP in carcinoma tissues is
determined using those samples.

The amount of P-LAP present in the tissues of carcinoma
patients can be determined by immunoassay, or more specifically,

by (a) contacting carcinoma tissues obtained from carcinoma patients with an anti-P-LAP antibody, followed by (b) measuring the intensity of the specific antigen-antibody reaction between P-LAP present in the carcinoma tissues and anti-P-LAP antibody.

5 When the streptavidin-biotin-peroxidase method, for example, is employed for the aforementioned immunoassay, the method may be carried out as follows.

 Firstly, deparaffinized carcinoma tissue samples are incubated with hydrogen peroxide to block endogenous peroxidase, and then further incubated with normal goat serum to block
10 non-specific binding. An anti-P-LAP antibody (primary antibody) is added thereto to effect antigen-antibody reaction, followed by addition of horseradish peroxidase-conjugated streptavidin for binding reaction. Conditions for the
15 antigen-antibody reaction and the binding reaction are not specifically limited, and may be similar to those usually employed for those types of reactions.

 A chromogenic substrate such as 3-amino-9-ethylcarbazole is acted on the complex obtained as above (complex of P-LAP, anti-P-LAP antibody, biotinylated goat anti-rabbit IgG and
20 peroxidase-conjugated streptavidin) in the presence of hydrogen peroxide to develop color, and counterstained with hematoxylin. The amount of P-LAP in the carcinoma tissues can be determined by analysis of the staining intensity of samples.

25 Enzymes other than peroxidase can be used as a marker for determination of the amount of P-LAP in carcinoma tissues as long as anti-P-LAP antibody bound to P-LAP can be detected by the method. Examples of such enzyme include malic dehydrogenase, staphylococcal nuclease, delta-5-steroid

isomerase, yeast alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, 5 glucose-6-phosphate dehydrogenase, glucoamylase, acetylcholineesterase and the like.

An assay kit can be provided by combining the aforementioned marker enzyme with an anti-P-LAP antibody. The kit optionally comprises a reagent for binding an anti-P-LAP 10 antibody with a marker enzyme, a reaction solution, a substrate, a substrate diluent and/or a reaction stop solution.

The marker may be a radioactive isotope, a fluorescent material, a chemiluminescent material, a bioluminescent material, etc. other than enzyme. These markers are attached 15 to desired molecules by a common technique conventionally used by those skilled in the art.

Next, the intensity of P-LAP immunoreactivity (intensity of the specific antigen-antibody reaction between P-LAP and anti-P-LAP antibody) in carcinoma tissues measured above is 20 correlated with prognosis of carcinoma, thereby prognosis of the carcinoma can be evaluated. The aforementioned process is explained below by using the case of endometrial endometrioid adenocarcinoma, including endometrioid adenocarcinoma, as an example.

25 The intensity of the specific antigen-antibody reaction between P-LAP and anti-P-LAP antibody, for example P-LAP immunostaining intensity, determined by the above immunoassay was scored on a three-tiered scale of negative (-), weakly positive (+) and strongly positive (++) , and the correlation

between the intensity and disease-free interval in endometrial endometrioid adenocarcinoma, including endometrioid adenocarcinoma, cases was examined. The results revealed that the disease-free survival rate for patients who had negative
5 or weakly positive intensity was significantly higher than that for patients who had strongly positive intensity. In addition, further studies showed that any of the characteristics including surgical stage of the disease, tumor grade (differentiation stage), myometrial invasion, vascular
10 infiltration and lymph node involvement, had a positive correlation with P-LAP immunostaining intensity.

Those facts indicate that P-LAP present in carcinoma tissues is a reliable prognostic marker for carcinoma. Therefore, prognostic evaluation of carcinoma can be achieved
15 when the amount of P-LAP present in carcinoma tissues obtained from carcinoma patients is determined by the aforementioned immunoassay using an anti-P-LAP antibody, and then the intensity of immunoreaction between P-LAP and anti-P-LAP antibody is correlated with prognosis of carcinoma.

20

Examples

The present invention will be further illustrated by way of the following Examples, although they do not limit the present invention.

25 Example 1

(1) Patients

Ninety-nine patients with endometrial endometrioid adenocarcinoma, including endometrial carcinoma and endometrioid adenocarcinoma, who had been surgically treated

at Nagoya University Hospital between January 1993 and December 2000 were included in this study. Diagnosis was established in all cases preoperatively by curettage of endometrium. Many patients were treated by radical hysterectomy with pelvic lymph
5 node dissections. However, in 21 patients, lymph node dissection was not performed because of their age and complications. All patients were staged according to the Federation of International Gynecology and Obstetrics (FIGO) classification, and their tumors were graded as well
10 differentiated (G1, $\leq 5\%$ solid components), moderately differentiated (G2, 6-50% solid components), or poorly differentiated (G3, $\geq 50\%$ solid components). In this study, the investigation was limited to endometrioid adenocarcinoma because of the small number of other histological types.
15 Adjuvant chemotherapy was administered to stage I patients with risk factors such as high histological grade and the like, and to all stage II, III, and IV patients. Two to three weeks after surgery, these patients were subjected to 4-6 cycles of cisplatin-based chemotherapy. Median follow-up time of
20 surviving patients was 55.3 months, ranging from 20 to 120 months. All subjects were examined every 3 months for the first 2 years and every 6 months thereafter.

The status of each stage is as follows.

25 Stage I: carcinoma tissue is present only in the endometrium

Stage II: carcinoma spreads to the uterine cervix beyond the endometrium

Stage III: carcinoma spreads outside of the uterus, but not outside of the small pelvis

Stage IV: carcinoma spreads beyond the small pelvis to other parts of the body, or reaches to the bladder or rectum

(2) P-LAP Immunostaining

5 Informed consent was obtained from each patient for sample use. All tissue samples were fixed in 10% formalin, embedded in paraffin, and routinely stained with hematoxylin and eosin for histological examination.

 Anti-human P-LAP polyclonal antibody was prepared
10 according to the method disclosed in PLACENTA, 2000, vol.21, p.628-634. Immunohistochemical staining was performed using the avidin-biotin immunoperoxidase technique (Histofine SAB-PO kit, Nichirei, Tokyo, Japan). Sections were cut at a thickness of 4 μ m, and immunostained by
15 streptavidin-biotin-peroxidase method. Areas containing viable carcinoma cells were confirmed by pathologists and selected. Deparaffinized sections in 0.01 M citrate buffer were treated three times for 5 minutes at 90 °C at 750 W with an H2500 microwave oven. Sections were incubated with 0.3%
20 hydrogen peroxide for 20 minutes and then further incubated with 10% normal goat serum. Anti-human P-LAP polyclonal antibody (0.4 μ g/section) was added to the tissue sections and incubated for 1 hour. The binding of the antibodies was followed with biotinylated goat anti-rabbit IgG and horseradish
25 peroxidase-conjugated streptavidin (Histofine SAB-PO, Nichirei, Tokyo, Japan). Chromogenic development was performed by immersion of the sections in 3-amino-9-ethylcarbazole (AEC, Nichirei, Tokyo, Japan). The slides were counterstained with Mayer's hematoxylin. To

assess the specificity of the reaction, there was a negative control in all cases. The negative control slide was prepared from the same tissue block. Immunostaining intensity was scored semi-quantitatively on a three-tiered scale (negative = -, weakly positive = +, strongly positive = ++) relative to the known positive and negative controls. Both routine hematoxylin and eosin slides and P-LAP reaction were reviewed independently by two pathologists.

10 (3) Statistical Analysis

The association between distribution of P-LAP and clinicopathologic characteristics were evaluated using the Fisher exact test or Chi-square test. The Kaplan-Meier method was used to summarize the distributions of disease-free interval. Log-rank test was used to evaluate individual factors with respect to these outcomes and the step-wise Cox proportional hazards model was used to assess multiple factors simultaneously. Patients who died without evidence of disease recurrence were censored as of the date of death. For comparison between two parameters, p less than 0.05 were considered significant.

(4) Results

The protein expression and cellular localization of P-LAP were analyzed by immunohistochemical staining of the tissue sections obtained from human endometrial endometrioid adenocarcinoma patients.

Fig. 1A shows the representative of P-LAP staining exhibited weakly positive in G1 endometrioid adenocarcinoma. Fig. 1B

shows the representative of strongly positive in G1 endometrioid adenocarcinoma. The strongest immunoreactivity for P-LAP was focused in the area with tumor cell invasion to the myometrial layer of G2 and G3 endometrioid adenocarcinoma (Fig. 1C, D). Normal endometrial cells were either negative or weakly positive.

The median age of the patients was 58.5 years (range, 36-75). Of 99 cases, 69 cases (69.7%) had specific P-LAP immunostaining. Weak staining was detected in 38 cases (38.4%) and strong staining in 31 cases (31.3%). Table 1 shows the relationships between P-LAP and some clinical characteristics, including histological grade, surgical stage of disease, myometrial invasion, lymph node involvement and vascular infiltration, etc.

Table 1

Characteristics	Total number of patient (%)	Number of patient showing strongly positive for P-LAP (%)	P
Age			
< 60	61 (61.6)	21 (34.4)	0.51
≥ 60	38 (38.4)	10 (26.3)	
Surgical Stage			
I - II	77 (77.8)	20 (26.0)	0.02 ^a
III - IV	22 (22.2)	11 (50.0)	
Grade (differentiation stage)			
1	55 (55.6)	4 (7.2)	< 0.01 ^b
2	29 (29.3)	18 (62)	
3	15 (15.1)	9 (60)	
Myometrial invasion			
none	11 (11.1)	0 (0)	0.01 ^b
1/2 or less	53 (53.5)	11 (20.8)	
1/2 or more	35 (35.4)	20 (57.1)	
Vascular infiltration			
negative	29 (29.3)	16 (55.2)	< 0.01 ^a
positive	70 (70.7)	15 (21.4)	
Lymph node involvement			
negative	10 (10.1)	7 (70)	< 0.01 ^b
positive	68 (68.7)	18 (26.4)	

a : Fisher exact test

b : Chi-square test

The present inventors found no significant correlation between P-LAP and patient age. P-LAP was established as strongly positive in 7.2% of G1 cases, in 62% of G2 cases, and in 60% of G3 cases. The present inventors found a positive correlation between P-LAP and histological grade ($p < 0.01$). The present inventors also found a positive correlation between P-LAP and surgical stage of disease ($p = 0.02$), muscular invasion

($p = 0.01$), lymph node involvement ($p < 0.01$), and vascular infiltration ($p < 0.01$).

Disease-free interval curves were drawn by Kaplan-Meier method to evaluate the prognostic impact of P-LAP expression.

5 The 10-year disease-free survival rates (DFS) of patients who had negative, weakly positive, and strongly positive expressions for P-LAP were 100%, 94.7%, and 47.3%, respectively (Fig. 2). Patients with strongly positive P-LAP staining showed a significantly lower disease-free interval compared

10 with patients showing negative or weakly positive P-LAP staining ($p < 0.01$). Furthermore, the analysis of disease-free interval was performed separately in early stage (stage I-II; $n=77$) and advanced stage (stage III-IV; $n=22$) patients. In the early stage patients, the 10-year disease-free survival rate

15 among those showing negative, weakly positive, and strongly positive expression for P-LAP were 100%, 96.6%, and 73.9%, respectively (Fig. 3). Patients with strongly positive P-LAP staining also showed a significantly lower disease-free interval compared with that of patients showing negative or

20 weakly positive P-LAP expression ($p < 0.05$). In the advanced stage, only one patient was negative for P-LAP, so this patient was included among patients with weakly positive P-LAP expression. The 10-year disease-free survival rates of patients who had negative or weakly positive expression for

25 P-LAP was 90%, and was 8.3% for those who had strongly positive expression with the difference being significant ($p < 0.01$) (Fig. 4).

Table 2 shows the univariate and multivariate analysis of disease-free interval according to the clinical

characteristics and P-LAP status.

Table 2

Parameter	Category	Univariate analysis <i>P</i>	Multivariate analysis		
			odds ratio	95 % CI	<i>P</i>
Age	< 60	0.07	1.02	0.95 - 1.08	0.55
	≥ 60				
Surgical stage	I / II	< 0.01	8.78	2.77 - 27.8	< 0.01
	III / IV				
P-LAP	Negative/Weakly positive	< 0.01	12.8	2.84 - 58.8	< 0.01
	Strongly positive				
Grade (differentiation stage)	1 / 2	0.02	-	-	-
	3				
Myometrial invasion	negative or 1/2 or less	0.14	-	-	-
	1/2 or more				
Vascular infiltration	Positive	0.06	-	-	-
	Negative				

95 % CI: 95 % confidence interval

Lymph node metastasis was excluded as a factor in these analyses since 21 patients did not undergo lymphadenectomy. Univariate analysis demonstrated that the following factors were significant: surgical stage, tumor grade (differentiation stage), and P-LAP expression. These factors were further analyzed by multivariate methods. Multivariate analysis demonstrated that P-LAP expression and surgical stage were independent prognostic factors.

Example 2

The prognostic impact of P-LAP expression on patients with ovarian carcinoma who had been surgically treated was

evaluated according to the same method as described in Example 1.

Of 61 patients (median age: 52.5, age range: 33-78), the numbers of patients showing negative, weakly positive, and
5 strongly positive expression for P-LAP are 12, 20, and 29, respectively.

The 10-year disease-free survival rates of patients who had negative expressions for P-LAP and patients who had weakly positive expressions for P-LAP were both 75%, while the 10-year
10 disease-free survival rate of patients who had strongly positive expressions for P-LAP was 48.3%. As mentioned above, patients showing strongly positive P-LAP staining showed a significantly lower disease-free survival rate compared with that of patients showing negative or weakly positive P-LAP
15 staining ($p < 0.05$).

Industrial Applicability

The reagent and the method for determination of the present invention can be used for prognostic evaluation of
20 carcinoma, thus they are useful in the field of pharmaceuticals and medical treatment.